SYNTHESIS AND AChE INHIBITING ACTIVITY OF 2, 4 SUBSTITUTED 6-PHENYL PYRIMIDINES

*[1]CRISTIAN PAZ, [2]MARTIN G. PETER, [2]BERND SCHMIDT, [1]JOSÉ BECERRA, [3]MARGARITA GUTIÉRREZ, [3]LUIS ASTUDILLO, [1]MARIO SILVA**

[1]Laboratorio de Química de Productos Naturales, Universidad de Concepción, Chile
[2]Institute of Chemistry, University of Potsdam, Germany
[3]Instituto de Química, Universidad de Talca, Chile
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ABSTRACT

Novel substituted pyrimidines were synthesized from methyl 2,4-dioxo-4-phenyl-butanoate (**I-A**) and urea, followed by Mitsunobu coupling of **I-A** with benzyl or allyl alcohol to give the corresponding 2-hydroxypyrimidine ethers in good yields. Saponification of **I-A**, followed by reaction with benzyl or allyl amines in the presence of TBTU yielded 2-hydroxy-6-phenyl-pyrimidine 4-carboxamides. AChE and BuChE assays revealed 2-hydroxy-6-phenyl-pyrimidine-4-carboxyallyamide as the most active compound, IC_{50}= 90 μM, with no inhibition of BuChE.

Keywords: Pyrimidines; inhibition AChE; mitsunobu; TBTU

INTRODUCTION

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease, is a progressive neurodegenerative disorder that affects regions of the brain that control cognition, memory, language, speech and awareness to one's physical surroundings[1]. Those alterations are associated with regional deficits in the cholinergic system. The development of acetylcholinesterase (AChE) inhibitor drugs has followed the finding that cholinergic pathways in the cerebral cortex and basal forebrain are compromised in AD[2] and the resultant cholinergic deficit contributes to the cognitive impairment of these patients[3]. Cholinesterase inhibitors (ChEIs) are considered to be valuable as a therapeutic target and they have become the main approach to symptomatic treatment; Donepezil, galantamine and rivastigmine are the first line pharmacotherapy for mild to moderate Alzheimer's disease[4]. The drugs have slightly different pharmacological properties, but they all work by inhibiting the breakdown of acetylcholine increasing the availability of acetylcholine in central synapses[4]. Acetylcholinesterase and butyrylcholinesterase (BuChE), the two forms of cholinesterase, co-exist ubiquitously throughout the body. They exhibit a high catalytic power and are very similar in structure and catalytic function. However, BuChE has a less defined role in biological processes, although it has been postulated that it acts as a detoxifying enzyme[5].

Looking for new active compounds we have isolated several natural alkaloids from *Aristotelia chilensis*[6], which have displayed broad range of pharmacology activities. Recently investigations have shown that substituted pyrimidines are potent ChE inhibitors[7] of Acetyl and Butylcholine. Therefore it seems interesting to synthesize 6-phenylpyrimidines as specific ChE, substituted at position 2 and 4 and evaluated their enzymatic activity.

EXPERIMENTAL

Melting points were determined on a Melting Point SMP10 (Stuart) and are uncorrected. The ^{1}H NMR spectra were determined using a Bruker ARX 300 instrument, operating at 300.1 MHz (^{1}H) and 75.5 MHz (^{13}C) or Bruker ARX 500 operating at 500 MHz (^{1}H) and 125 MHz (^{13}C). High-Resolution ESI mass spectra (HR-MS) were measured on a Q-TOF mass spectrometer Micromass (Manchester). EI low-resolution MS spectra were measured on Trace DSQII GC/MS-system Axel Semrau GmbH & Co). Column chromatography was performed using Merck silica gel 60 (0.063–0.200 mm). TLC was carried out on a Merck silica gel 60 PF254. Solvents used in this study were distilled prior to use and dried over appropriate drying agents.

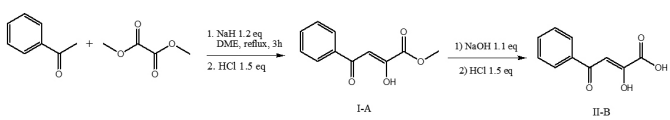
Enzyme assays

The *in vitro* measurement of AChE and BuChE inhibition were carried out using a colorimetric method[8] adapted to 96-well microtiter plates[9]. The AChE was obtained from *Electrophorus electricus* (C3389-500UN), type VI-S, Sigma Chemical Co., St. Louis, MO), and BuChE from equine serum (C1057-1KU, Sigma Chemical Co., St. Louis, MO). Fifty μL of test sample dissolved in 100 mM phosphate buffer pH 7.6, and 50 μL, of AChE or BuChE solution (final concentration of 0.03 U/mL and 0.01 U/mL, respectively) were added to each well, and the plates were pre-incubated for 30 min at room temperature. After the pre-incubation period, acetylthiocholine iodide (or butyrylthiocholine iodide) was added to a final concentration of 200 μM. 5,5`-Dithio-bis(2-nitrobenzoic acid) (DTNB) was used for the measurement of cholinesterase activity. The hydrolysis of ACh or BCh was monitored by following the formation of the yellow 5-thio-2-nitrobenzoate anion. The absorbance was read in a Thermo Multiskan Ex Instrument microplate reader at 405 nm after 3 min. The enzyme activity was calculated as a percentage compared to a control using only the buffer and enzyme solution. The compounds were assessed in the dilution interval of 500-15.63 μg/mL, and the alkaloid galanthamine was used as the reference compound. The ChEs inhibitory activity of each compounds was expressed in terms of the IC_{50} value (μg/mL and μM required to inhibit the hydrolysis of the substrate by 50%), as calculated from the dose-response curve.

RESULTS AND DISCUSSION

2, 4-Dioxobutanoates are interesting starting materials for the synthesis of ligands and organometallic complexes of anti-HIV-1 integrase[10]. Amides and hydrazides of 2, 4-dioxobutanoic acid possess antimicrobial and analgesic activity[11]. The β diketone I-A has been synthesized in 84% yield from the acetophenone and dimethyloxalate in dimethoxyethane, using NaH as base, scheme 1. Compound I-A shows an interchangeable proton at 15.83 ppm and just one ketone carbon at 190.81 ppm suggesting that I-A occurs only as the β-hydroxyketone.

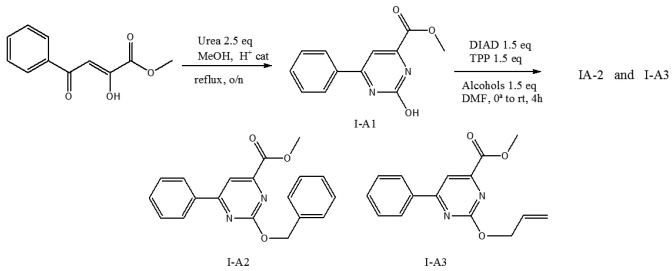
Hydrolysis of I-A in aqueous NaOH for 10-15 min gave the corresponding acid II-B in 80% yield. II-B is known to possess promising activity against HIV-1 integrase[7] and slow-binding inhibition of KDPG aldolase[12] which is a target for new bacteriostatic or bactericidal drugs. Other reaction conditions, such as NaOH in MeOH, afforded II-B in a yield of ca. 50%, together with unreacted starting material.



Scheme 1

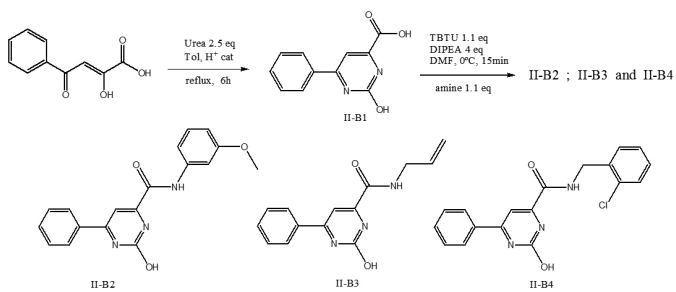
Pyrimidine I-A1 was synthesized from β-hydroxy ketone I-A in 78% yield, using an excess of urea in MeOH under reflux in the presence of catalytic amounts of H_2SO_4. Ethers I-A2 and I-A3 were obtained from I-A1 under Mitsunobu conditions, using diisopropylazodicarboxylate (DIAD) and Triphenylphosphine in dimethylformamide. Column chromatography afforded the products in ca. 87% yield, scheme 2.

e-mail: njsilva@udec.cl



Scheme 2

Reaction of 4-phenyl-2,4-dioxobutanoic acid (II-B) with an excess of urea and catalytic amounts of H_2SO_4 in refluxing toluene gave 2-hydroxy-6-phenylpyrimidine-4-carboxylic acid (II-BI) in yields of ca. 55%. II-BI precipitated after the reaction as a white solid, soluble in dimethylsulfoxide or dimethylformamide. Amide coupling of II-B1 with benzyl or allyl amines with O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumtetrafluoroborate (TBTU) in DMF gave compounds II-B2, II-B3, and II-B4, respectively, in ca. 40% yield, scheme 3. Attempts to prepare secondary amides with diethyl- or dibenzylamine were unsuccessful, as only traces of product were detected



Scheme 3

Compounds I-A2; I-A3; II-B2; II-B3; II-B4, as well as pyrimidines I-A1 and II-B1 were evaluated as inhibitors of AChE (Table 1). All amides showed higher activity than pyrimidine carboxylic acid, II-B1. The most active compound was the allyl amide II-B3 with an IC_{50} of 90.1 μM. All compounds were also tested with BuChE, but did not show significant inhibition (IC_{50} > 1000 μM).

Table 1. Activity of 6-phenyl pyrimidine 2,4 substituted toward the enzyme AChE.

Entry	Compound	AChE IC_{50} mg/mL	μM
1	I-A1	0.024	104.3
2	I-A2	0.089	278.0
3	I-A3	0.032	118.0
4	II-B1	0.081	375.0
5	II-B2	0.059	184.0
6	II-B3	0.023	90.1
7	II-B4	0.063	185.8
*Galantamine		$1.1x10^{-3}$	3.0

* Internal control, n=3.

(*Z*)-Methyl 2-hydroxy-4-oxo-4-phenylbut-2-enoate (I-A): A mixture of acetophenone (5g, 41.7 mmol) and dimethyloxalate (5.9g, 50 mmol) were stirred in 150mL of anhydrous dimethoxyethane, then sodium hydride (1.2g, 50 mmol) was added. The mixture was stirred under reflux for 4 h. The reaction was cooled and quenched with water 100mL and HCl until pH 2. The mixture was extracted 3 times with EtOAc, and the organic layer was evaporated in vacuo. The compound precipitated as a white solid (7.2g, 84%). ^{1}H-NMR ($CDCl_3$, 300 MHz) ppm: 3.94 (3H, s); 7.10 (1H, s); 7.50 (2H, m); 7.61 (1H, m); 8.00 (2H, m), 15.83 (1H, s, interchangeable). ^{13}C-NMR ($CDCl_3$, 75 MHz) ppm: 55.41; 99.45; 127.55; 129.16; 130.19; 135.16; 162.61; 169.09; 190.81. EI-MS: 206

Methyl 2-hydroxy-6-phenylpyrimidine-4-carboxylate (I-A1): A mixture of compound I-A (3.0g, 14.6 mmol) and urea (2.8g, 47 mmol) were dissolved in 100mL of methanol and ca. 100 μL of conc. sulfuric acid was added. The solution was stirred overnight. The product precipitated as a white solid (2.8g, 78%) mp = 195-196ºC. ^{1}H-NMR (DMSO-d6, 500 MHz) ppm: 3.91 (3H, s); 7.59 (3H, m); 7.72 (1H, s); 8.13 (2H, d, J= 7.2 Hz); 12.47 (1H, s, br). ^{13}C-NMR (DMSO-d6, 125 MHz) ppm: 53.50; 105.98; 127.92; 129.51; 132.40; 135.23; 155.43; 162.54; 163.78; 168.22.

Methyl 2-(benzyloxy)-6-phenylpyrimidine-4-carboxylate (I-A2): DIAD (1.49g, 7.4mmol) in 10mL of dry THF was cooled in an ice bath, then triphenylphospine (1.93g, 7.4mmol) dissolved in 10 mL of THF was added, the mixture was stirred for 15 min, during which a white solid was formed. A mixture of I-A1 (1.13g, 4.9mmol) and benzyl alcohol (0.78g, 7.2mmol) dissolved in 15mL of dry dimethylformamide was added to the suspension. The reaction was stirred for 5 h from 0 ºC to room temperature. The solvents were removed in vacuo and the crude product was purified by silica gel column with hexane/EtOAc 30%. White solid (1.35g, 86%) mp = 98°C. ^{1}H-NMR ($CDCl_3$, 300 MHz) ppm: 4.03 (3H, s); 5.60 (2H, s); 7.27-7.42 (3H, m); 7.46-7.62 (5H, m); 8.07 (1H, s); 8.16 (2H, dd, J= 2.1, 7.6 Hz). ^{13}C-NMR ($CDCl_3$, 75 MHz) ppm: 53.30; 69.80; 110.59; 127.63; 128.22; 129.12; 131.87; 135.98; 136.45; 158.01; 164.96; 165.87; 168.78. ESI-MS: M+1: 321.1137

Methyl 2-(allyloxy)-6-phenylpyrimidine-4-carboxylate (I-A3): The compound was prepared following the same procedure as described for I-A2, using allyl alcohol (0.427g, 7.3mmol). The crude product was purified by CC with hexane-ethyl acetate 20%, giving a white solid (1.15 g, 87%) mp = 102 - 103°C. ^{1}H-NMR (MeOD, 300 MHz) ppm: 4.00 (3H, s); 4.91 (2H, s); 5.25 (2H, m); 6.00 (1H, m); 7.44 (1H, s); 7.57 (3H, m); 8.15 (2H, m). ^{13}C-NMR (MeOD, 75 MHz) ppm: 43.14; 52.92; 103.29; 117.69; 127.74; 128.69; 131.95; 132.44; 135.13; 148.32; 157.10; 161.52; 171.68. ESI-MS: M+1: 271.1389.

(*Z*)-2-Hydroxy-4-oxo-4-phenylbut-2-enoic acid (II-B): Hydrolysis of I-A (5.5g, 26.7mmol) was carried out with NaOH (1.1g, 26.7mmol) in 20mL of water and stirring at 50ºC for 15 min. The reaction was filtered and the rection mixture was extracted three times with 10 mL of ethyl acetate. The water layer was acidified with HCl until pH 2. The product precipitated as a white solid, it was filtered off and dried (4.1g, 80%) , mp = 160ºC. ^{1}H-NMR ($CDCl_3$, 300 MHz) ppm: 6.75 (1H, br); 7.52 (2H, m); 7.53 (1H, d, J= 4.2 Hz); 7.87 (2H, d, J= 4.5 Hz). ^{13}C-NMR ($CDCl_3$, 75 MHz) ppm: 98.04; 127.48; 128.00; 128.42; 128.90; 133.05; 135.44; 163.81. EI-MS: 192

2-Hydroxy-6-phenylpyrimidine-4-carboxylic acid (II-B1): A mixture of II-B (1.0g, 5.2mmol), urea (0.98g, 16.3mmol) and ca. 100 μL of conc. sulfuric acid were dissolved in 100 mL of toluene and stirred overnight, during which a brown solid precipitated, which was filtered off and washed with cold methanol. White solid (0.7g, 62.2%), decomposes at ca. 215°C, ^{1}H-NMR (DMSO d6, 300 MHz) ppm: 7.25-7.60 (4H, m, overlap); 8.13 (2H, dd, J= 2; 6 Mz). ^{13}C-NMR (DMSO d6, 75 MHz) ppm: 103.8; 128.40; 129.83; 132.75; 136.14; 154.67; 160.61; 164.00; 169.86. ESI-MS: M+1: 217.0711

2-Hydroxy-N-(3-metoxyphenyl)-6-phenylpyrimidine-4-carboxamide (II-B2): A mixture of 2-hydroxy-6-phenylpyrimidine-4-carcarboxylic acid (150mg, 0.69mmol) and N,N-Diisopropylethylamine (180mg, 1.4mmol) were dissolved in 10mL of dry DMF, then O-(Benzotriazol-1-yl)-N,N,N',N'-tetram ethyluroniumtetrafluoroborate (TBTU) (268 mg, 0.83mmol) was added. The mixture was stirred for 30 min at 0ºC, and 3-methoxyaniline (94mg, 0.76mmol) was added. The reaction was stirred for 3 h, then the reaction was quenched with H_2O, and the DMF was removed in vacuo (5 mmbar and 60ºC), the crude was purified by CC with hexane-ethyl acetate 50%. Yellow solid (100mg, 45%), decomposes at ca. 205ºC. ^{1}H-NMR (DMSO d6, 300 MHz) ppm: 3.77 (3H, s); 6.76 (1H, dd, J= 3.0; 9.0 Hz); 7.31 (1H, dd, J= 9; 15 Hz); 7.4 – 7.7 (6H, m, overlap); 7.80 (2H, d, J= 9 Hz); 10.51 (1H, s). ^{13}C-NMR (DMSO d6, 75 MHz) ppm: 55.95; 107.03; 110.45; 110.95; 113.48; 120.86; 126.23; 128.19; 128.36; 129.93; 130.42; 132.75; 134.87139.87; 143.75; 160.30. ESI-MS, M+1: 322.1339

N-Allyl-2-hydroxy-6-phenylpyrimidine-4-carboxamide (II-B3): The compound was prepared following the same procedure as described for II-B2, using allylamine (87mg, 2.0mmol). White solid (140 mg, 40%), decomposes at ca. 150°C. ^{1}H-NMR (MeOD, 300 MHz) ppm: 4.04 (2H, m); 5.20 (2H, m); 5.94 (1H, m); 7.48 – 7.65 (4H, m); 7.96 (2H, d, J= 3, 6 Hz). ^{13}C-NMR (MeOD, 75 MHz) ppm: 43.14; 111.57; 116.90; 118.82; 127.28; 128.42; 128.80; 130.54; 133.55; 134.97; 164.39; 173.15. ESI-MS: M+1: 256.1131

N-(2-Chlorobenzyl)-2-hydroxy-6-phenylpyrimidine-4-carboxamide (II-B4): The compound was prepared following the same procedure as described for IIB2, using 2-chlorobenzyl amine (216 mg, 1.53mmol). White

solid (200mg, 43%), decomposes at ca. 205 ºC. ^{1}H-NMR (DMSO d6, 300 MHz) ppm: 4.58 (2H, d, J= 3 Hz); 7.29-7.35 (2H, m); 7.42 (1H, t, J= 3Hz); 7.53 – 7.60 (4H, m); 7.73 (1H, d, J= 6 Hz); 8.00 (1H, d, J= 3 Hz); 8.07 (1H, d, J= 3 Hz); 9.38 (1H, s). ^{13}C-NMR (DMSO d6, 75 MHz) ppm: 41.01; 110.08; 119.60; 125. 00; 127.67; 127.79; 127.95; 128.29; 129.08; 129.18; 129.55; 129.61; 133.00; 136.19; 143.30; 163.37. ESI-MS: M+1: 340.1096

CONCLUSIONS

The investigation described in this communication reports the synthesis of substituted pyrimidines in few steps with goods yields and simplicity of operations. Separately we modified the pyrimidine substituent at position 2 through Mitsunobu reaction and the carboxy group at position 4 by amidation. All compounds were evaluated as AChE inhibitors showing that position 4 is the most important for enzyme-inhibition. N-Allyl-2-hydroxy-6-phenylpyrimidine-4-carboxamide (II-B3) is the most active compound against AChE with IC_{50} 90.1µM. It is highly selective for AChE, not showing activity against BuChE.

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